Time-Dependent Apoptotic Development and Pro-apoptotic Genes Expression in Rat Heart After Myocardial Infarction

Yi Zhun Zhu[1, 3,]*, Yi-Chun Zhu[2, 3], Zhong Jing Wang[1], Qing Lu[1], How Sung Lee[1] and Thomas Unger[3]

[1]Department of Pharmacology, National University of Singapore, 10 Kent Ridge Crescent, Singapore 119260
[2]Department of Physiology, Shanghai Medical University, Shanghai, China
[3]Department of Pharmacology, University of Kiel, 24105 Kiel, Germany

ABSTRACT—We investigated the apoptotic development and apoptotic-related gene expression after myocardial infarction (MI) at different time points in the current study. Bax gene expression was increased at 12 h after MI and peaked at 24 h. Fas gene started to over-express at 12 h after MI as well but it reached maximum at 72 h. In the MI groups, strongest staining of apoptosis was detected in rats 3 days post operation. Our results demonstrate that apoptotic development after MI is time dependent in the ischemic area and there could be some linkage with the over expression of angiotensin II receptors post MI.

Keywords: Myocardial infarction, Gene expression, Apoptosis

Myocardial infarction (MI) remains the leading cause of death from cardiovascular diseases over the past decades. Recent accumulating evidence has shown that apoptosis can result from mild cellular injuries such as hypoxia (1) and ischemia (2). MI was shown to lead to apoptosis in cardiomyocytes (3), whereas cell destruction was caused mainly by necrosis. Both Fas (4) and Bax (5) have been shown to be directly involved in cell death after myocardial ischemia. Antiapoptotic interventions can delay ischemic myocardial damage in experiments. A caspase inhibitor has been reported to be effective in reducing myocardial reperfusion injury, an action that was partially attributed to attenuation of cardiomyocyte apoptosis (6). Gene expression of both angiotensin (ANG)-receptor subtypes AT1 and AT2 after MI is time-dependent (7, 8), and there is growing interest in ANG II receptor-mediated apoptosis. In a recent study in an animal model of stroke, we reported that up-regulation of angiotensin AT_2 receptor may be involved in the apoptosis of tissue repair after stroke (2). It is still unclear whether there is any linkage of increased ANG II receptors gene expression with apoptosis after MI. We therefore investigated pro-apoptotic genes (Fas and Bax) expression and the apoptotic development at different time points after MI.

Experiments were performed on male Wistar rats weighing 250 and 300 g. MI was induced by ligation of the left

*Corresponding author.   FAX: +65-773-0579
 E-mail: phczhuyz@nus.edu.sg

anterior descending coronary artery as described previously (8). This animal model was approved by the ethical committee and conformed to internationally accepted ethical standards. For investigating possible apoptosis-related gene expression, half of the MI-induced rats were sacrificed at 12, 24 and 72 h after surgery (n = 6). Sham-operated rats (n = 5 at each time point) were taken as the control. Another group of rats was prepared for apoptotic assay. TUNEL (terminal deoxynucleotidytransferase-mediated dUTP-biotin nick end labeling) was applied for detecting apoptotic development after MI. At 1, 3, 7 and 14 days after MI (n = 3 at each time points) or sham operation (n = 3), the animals were decapitated and hearts were prepared for apoptotic (DNA fragmentation) assay.

For isolating total RNA, the left ventricle, septum and right ventricle were separated and homogenized in a 10-fold volume (wt/vol) of ice-cold Trizol reagent (Gibco BRL, Rockville, USA) using a Polytron homogenizer (Janke & Kunkel, Kelheim, Germany). Total RNA was extracted according to the manufacturer's instructions. Five micrograms total RNA from the different parts of hearts was reverse-transcribed into first-strand complementary DNA (fs cDNA) using oligo-dT primers (Gibcol BRL). The fs cDNA (1 μl) was amplified with 2.5 units of Taq DNA polymerase (Promega, Madison, USA) by PCR. The expression of the 'house keeping' gene, β-actin mRNA, was considered as an internal standard. The following primer sequences were used: sense 5'-GCAGGGAG GATGGCTGGGGGAGA-3', antisense 5'-TCCAGACAAG

CAGCCGCTCACG-3' for Bax and sense 5'-AACAT GAGAACATCCTGTGCC-3', antisense 5'-TCCCTGCTC ATGATGTCTACC-3' for Fas gene, and sense 5'-ATCTG GCACCACACCTTCTACAATGAGCTGCG-3', antisense 5'-CGT CATACTCCTGCTTGCTGATCCACATCTGC-3' for β-actin. PCR was run 25 cycles for β-actin as well as for cDNA for Bax and Fas in a thermocycler (Model 2400; Perkin Elmer, Norwalk, CT, USA). Three-step PCR of denaturing, annealing and extension reactions was run at 94°C for 1 min; at 60°C (β-actin), at 64°C (Bax) or at 56°C (Fas) for 1 min; and 72°C for 1 min, respectively. The PCR amplifications were linear. The primers were designed to cross an intron to prevent amplification of contaminating genomic DNA. PCR products were analyzed and quantified by the Scion Imaging system.

ApoAlert® DNA fragmentation assay kit (Clontech, Palo Alto, USA) was used to detect apoptosis-induced nuclear DNA fragmentation via a fluorescence assay. The assay is based on TUNEL which was recently published (2).

Results are presented as mean ± S.E.M. All data were analyzed by a one-way ANOVA for independent evaluations over all groups. In case of a P-value being less than 0.05, difference between individual groups were analyzed using student's t-test.

To determine the Bax and Fas gene expression in sham operated rats and coronary artery ligated rats at different time points post MI, 1 μg of total RNA from each rat was pooled together according to its time point (n = 5 or 6). Pooled total RNA was then reverse transcribed into cDNA for subsequent PCR amplification. An additional negative control was carried out in the absence of tissue material to exclude any possibility of sample contamination.

No Bax gene expression was found in the sham operated rats and rats 12-h post MI. An over-expressed Bax gene was detected at 24 h, but its level declined at 72 h after MI. Gene expression level of Bax was 4.3-fold (left ventricle), 1.7-fold (septum and right ventricle) at 24 h compared to that at 72 h after MI (Fig. 1A).

Fas gene was expressed in a similar manner, but the peak was delayed to 72 h. Unlike the Bax gene, Fas gene expression was detected in the sham-operated rats and started to over-express at 12 h (1.5-fold in the left ventricle, 2.2-fold in the septum and 2.4-fold in the right ventricle) after MI compared to sham-operated rats. A significant over-expression of Fas gene was observed with 1.9-fold in the left ventricle and 4.7-fold in the septum compared to sham-operated rats at 72 h (Fig. 1B).

The apoptotic cells were identified with TUNEL staining. In paraffin section of rat heart after MI, the normal architecture was absent in the infarct zone of left ventricle, while no change was observed in the sham operated rats viewed with HE staining under a microscope. Frozen sections were prepared immediately after sacrifice of the
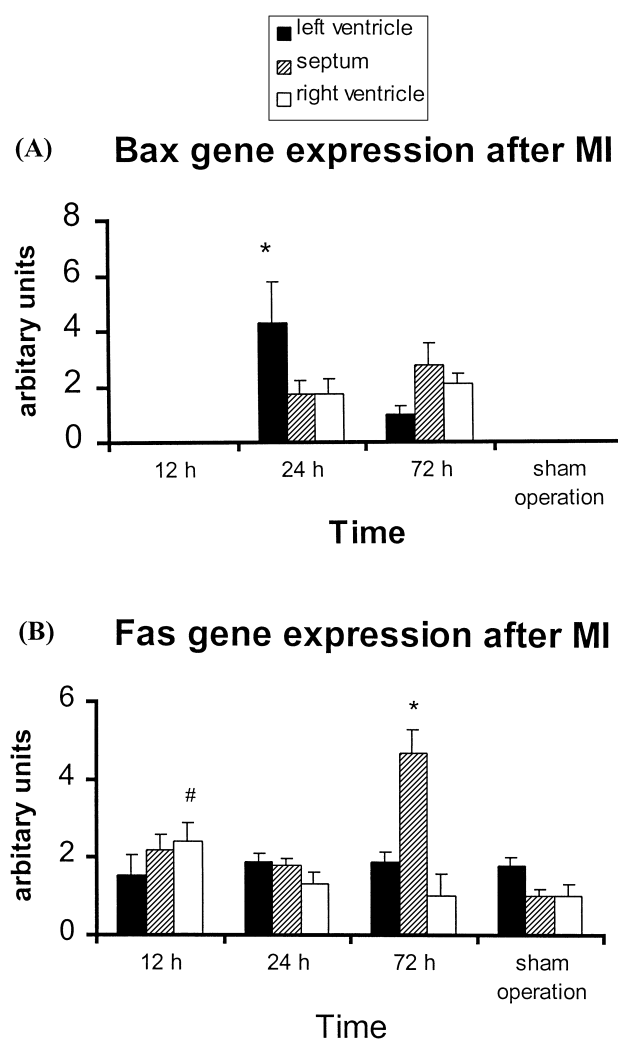

(A) Bax gene expression after MI

(B) Fas gene expression after MI

Fig. 1. The quantified mRNA levels of Fas and Bax at different time points of rats in left ventricle, septum and right ventricle after myocardial infarction (MI). *$P<0.001$ vs sham-operated rats, #$P<0.05$ vs sham-operated rats.

animal for ApoAlert® (TUNEL) staining. The slides were viewed and photographed by using a fluorescent Leica microscope. Apoptosis was detected in the infarcted areas. No apoptosis was detected in the sham operated rats. In the MI groups, the strongest staining of apoptosis was detected in rats 3 days post operation. Weak stainings were found 1-day and 7-day post MI. Very few apoptotic cells were detected in the rats 2 weeks after MI (Fig. 2).

MI produces an immediate loss of contractile function of the supplied tissue and a redistribution of ventricular workload to the surviving myocardium. Such an event rapidly evokes a reaction between infarcted and non-infarcted myocardium that is referred to as ventricular remodeling. Time-dependent architectural alterations in the non-infarcted region as well as in the infarcted zone have been observed. Gene expression of ANG II AT1 and AT2
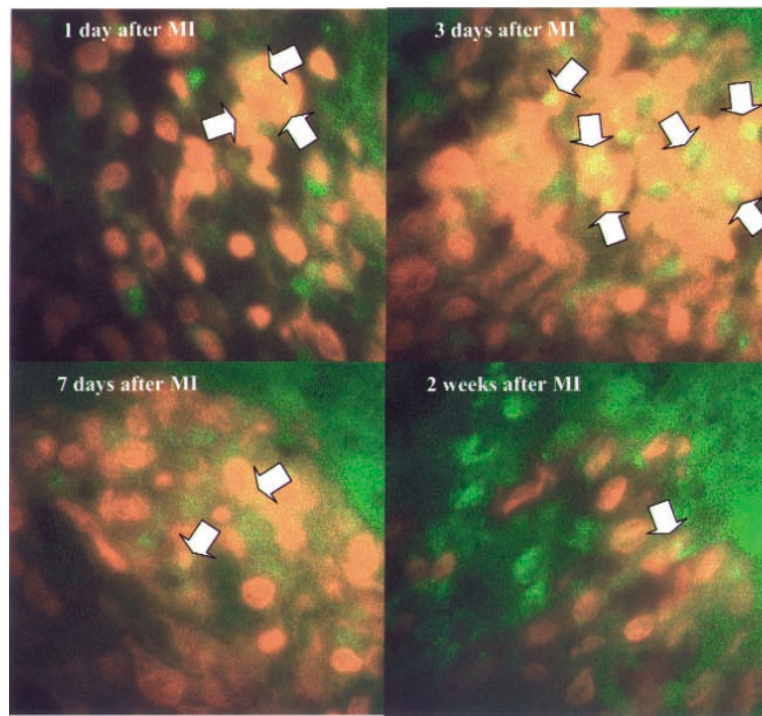
Fig. 2. Apoptotic staining in left ventricles on days 1, 3, 7 and 14 post myocardial infarction (MI). Apoptotic cells (arrows) exhibited strong nuclear green fluorescence on the same field of infarcted myocardium. The scale bar indicates 125 μm.

receptors has been reported to be changed in the ischemic animal models (2, 7–9). Up-regulation of AT2 receptor was shown to be involved in the apoptosis of tissue repair after stroke (2). Our current results have shown strong evidence for over-expression of pro-apoptotic genes and further indicate that apoptosis was time dependent after MI. The up-regulation of pro-apoptotic genes was found in the left ventricle, septum and right ventricle, while apoptosis was only detected in the necrotic areas. As we know, necrosis does not indicate a form of cell death but refers to changes secondary to cell death by any mechanism, including apoptosis (10). Cell death in the necrotic tissue of the left ventricle is not caused by programmed cell death but accidental cell death (11). However, Wyllie and co-workers proposed that necrosis is the term currently used for non-apoptotic, accidental cell death (1). Necrosis can occur after apoptosis (10). On the other hand, post-apoptotic change was also considered as secondary necrosis because apoptotic cell bodies can incur extracellular breakdown. Unlike necrosis, apoptosis may be either beneficial or harmful depending upon coexisting circumstances. In an animal model of MI, it was reported that cell death in the first couple of days post MI was entirely by apoptosis; in the next few days, a mixture of apoptosis and necrosis; and all cell death after that was essentially by necrosis (12). In an examination of human hearts of individuals dying of acute MI, both apoptosis and necrosis were found to be present in every case (13). Our current studies demonstrated for the first time the time-course of apoptotic development as well as pro-apoptotic genes expression after MI. It is still unclear whether apoptosis causes necrosis or necrosis causes apoptosis after MI (13). It seems like in our experiments that apoptotic development accompanied necrosis, where apoptosis appeared even earlier. There is some evidence from human studies to support our findings. These studies reported that infarcted myocardium showed a DNA ladder on agarose gel electrophoresis, indicating apoptosis concomitant with necrosis (14). Combined with the recent reported finding of AT1 receptor related to apoptosis in the rat infarction model (15), our results demonstrate that Fas and Bax genes are directly involved in apoptosis in the acute phase of MI. Apoptotic cells were mainly located in the necrotic area of an infarcted heart. Apoptotic development after MI is time-dependent. Combined with the recent reported finding of AT1 receptor related to apoptosis in the rat infarction model (15), we could therefore conclude that there would be some linkages between apoptosis and over expression of both ANG II AT1 and AT2 receptor subtypes post MI. Apoptosis may be an additional mechanism of cell death after MI.

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